REMARKS

Claims 39-43, and 46-67 constitute the pending claims in the present application. Issues raised by the Examiner will be addressed below in the order they appear in the prior Office Action. Applicants respectfully request reconsideration in view of the following remarks.

1a. The Office Action noted that the response filed July 24, 2002 did not include claims 44 and 45 in the listing of all claims being examined, and that there was no amendment instruction requesting cancellation of claims 44 and 45. Applicants respectfully point out that the first paragraph of the Remarks section stated that "Applicants have cancelled claims 1-28, 44 and 45." In any case, Applicants hereby formally request cancellation of claims 44 and 45. The cancellations have been made solely to expedite prosecution. Applicants reserve the right to pursue claims of similar or differing scope to the cancelled claims at a later time.

1b. Applicants have taken steps to conform to 37 CFR 1.121(b)(1)(iii) and (c)(1)(ii) as requested by the Examiner. Applicants have amended claim 38 by capitalizing the "a" at the beginning of the claim. Applicants assert that this amendment does not narrow the scope of the claim. Applicants have also amended claim 66 to harmonize the clean and marked-up claims presented in the response filed July 24, 2002. Applicants thank the Examiner for pointing out these informalities.

2. Applicants acknowledge approval of the declaration signed by Inventor Bachovchin.

3. Applicants have herein corrected their claim to priority by amending the specification. Thus, Applicants assert that there is proper co-pendency between the two applications. Applicants respectfully request reconsideration of the Examiner's objection.

4. Claims 42 and 43 are rejected under 35 U.S.C. 112, first paragraph, for lack of written description. Applicants have cancelled claim 43. Applicants respectfully traverse the rejection of claim 42.

Claim 42 is directed to a method for modifying glucose metabolism of a glucose intolerant animal, wherein, the glucose intolerance in the animal is a result of a deletion or disruption of the gene encoding for a glucagon type peptide 1 (GLP-1) receptor. In their July 24, 2002 response, Applicants cited to the specification on page 51, line 6 as support for the claim. The citation was meant to encompass both the Gallwitz et al. article and the actual experiment Applicants conducted on GLP-1 receptor -/- transgenic mice. Applicants point out that the journal citation is correct. Applicants have attached herein, as Appendix I, a copy the MEDLINE citation for the article.

In any case, Applicants assert that both Gallwitz et al., and Applicant's own experiment shown in Figure 4, is sufficiently enabling of claim 42. As the title of their article points out, Gallwitz et al. teach that disruption of the GLP-1 receptor gene leads to insulinoma, which is a form of glucose intolerance. Applicants show in Figure 4 that a GLP-1 receptor -/- transgenic mice has high blood glucose which is ameliorated by administration of a compound of the present invention. As such, Applicants submit that the specification describes the subject matter of claim 42 in such a way as to reasonably convey to one skilled in the relevant art that Applicants, at the time the application was filed, had possession of what is claimed in claim 42.

The Office Action further noted that the specification is further limited to mice, and that "there is no indication that a GLP-1 receptor gene deletion or disruption exists in glucose intolerant animals in general." Applicants respectfully submit that claim 42 is sufficiently enabled by Gallwitz et al. and the Applicants own experiments with GLP-1 receptor -/- transgenic mice. Further, Applicants point out that the mouse system is a reasonable model for studying similar conditions in other animals, such as humans. Applicants submit one skilled in the relevant art, having read the present application, can determine whether an animal has a non-functional GLP-1 receptor gene, whether the animal is glucose intolerant and how to administer the compounds of the present invention to modify this glucose intolerance. As such, Applicants submit that the specification has adequate written descriptive support for claim 42. Accordingly, in view of the arguments presented above, Applicants respectfully request reconsideration and removal of the rejection.

5. Claims 39-49 and 51-67 are rejected under 35 U.S.C. 112, second paragraph, over the use of the terms "a Ki in the nanomolar range" in claims 38-40, over lack of antecedent basis for the term "the glucagon type peptide" in claim 43, and the use of the variable R_1 in claim 66. Applicants have amended claim 38 read "a Ki of less than about 10 nM." Applicants have amended the claim solely to expedite prosecution. Applicants reserve the right to pursue claims of similar or same scope as the claim prior to amendment. Applicants have cancelled claim 43. Finally, Applicants have amended claim 66 to remove the variable R_1. Applicants submit that this amendment does not narrow the scope of the claim. Applicants have further amended claim 66 to include definitions for variables R_2, Y_1, and Y_2. Applicants respectfully submit that one skilled in the relevant art, having read the specification, would have identified the definitions for these variables, and thus, would not have been confused as to the scope of the claims. In view of these amendments and cancellations, Applicants respectfully request reconsideration and removal of the rejection.

6. Applicants have herein corrected the informalities in claims 38. Applicants have capitalized the beginning letter of the claim 38. Applicants submit that an "or" is already present in claim 65 at the place cited by the Office Action, i.e., page 15, line 13 of the response filed on July 24,2002.

7. Applicants submit that the rejection of claims 44 and 45 has been rendered moot in light of Applicant's cancellation of the claims.

8. Claims 38-67 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-14 and 16-37 of the copending Application No. 09/601,432. Applicants will address this issue when the rejection is no longer provisional.

9. Claims 38-67 are provisionally rejected under the judicially crated doctrine of obviousness-type double patenting as being unpatentable over claims 37-131 of co-pending Application No. 10/190,267. Applicants will address this issue when the rejection is no longer provisional.

10. In points 12-13 the Office Action posed 103 rejections based in part on the disclosure of Deacon et al. Applicants respectfully traverse these rejections. Applicants assert that the instant application is a continuation of the PCT Application US99/02294 filed on 02/02/99, which in turn claims priority to the US Application 60/073,409, filed on 02/02/98. Applicants have entered an amendment to the specification to establish the earlier priority date, proper copendency. Applicants assert that in the absence of Deacon et al. none of the 103 rejections in points 12 and 13 can stand. Accordingly, Applicants respectfully request reconsideration and removal of these rejection.

11. In points 14 and 15 claims 38-41, 44-52, 54-57, 59, 60, 63 and 66 are rejected under 35 U.S.C. 103(a) as being obvious in view of WO Patent Application 98/25644 by Drucker (hereafter referred to as the '644 Application), and its U.S. equivalent. Applicants respectfully traverse the rejection.

Applicants assert that the cited art does not render obvious the instant claims because the reference teaches co-administration of ***GLP-2*** with Pro(boro)Pro. Claims 38-41, 44-52, 54-57, 59, 60, 63 and 66 are not directed to co-administration of GLP-2 with the claimed compounds. As such the '644 does not teach or suggest as the Office Action alleges "the same active agents being administered to the same animals according to the same method steps". Thus, it does not render obvious the instant claims. Accordingly, Applicants respectfully request reconsideration and removal of the rejections.

13. Claims 38-41, 44-52, 54-57, 59, 60, 63 and 66 are rejected under 35 U.S.C. 102(a) and (e) as being anticipated by Villhauer. Applicants respectfully traverse the rejection to the extent that it is maintained over the amendments entered.

As to claims 38-41, Applicants assert that Villhauer does not provide an enabled disclosure which teaches treating glucose intolerant animals. Villhauer's opening statements in the Abstract notwithstanding, Applicants point out that for a prior art to be anticipatory it has to have an enabling disclosure.(See MPEP 2131.01) Unlike Applicants who have demonstrated the efficacy of the instantly claimed compounds by treating animals with glucose intolerance, Villhauer provides no such enabled disclosure. The only in vivo experiment Villhauer provides is the administration of compounds to

normal male Sprague-Dawley rats and measurement of early insulin response. (See col. 9, line 66 to col 10 line 28) There is no indication that these rats are glucose intolerant, have diabetes, or are non-insulin dependent diabetics. In contrast, Applicants show in Figure 4 that a GLP-1 receptor -/- transgenic mice has high blood glucose which is ameliorated by administration of a compound of the present invention. The animal models used by Applicants are truly glucose intolerant. As such, Applicants assert that Villhauer does not teach all the elements of the instant claims, and therefore fails to anticipate the claims.

Applicants further point out that the remaining claims, as amended, do not include compounds covered by Villhauer. All of Villhauer's compounds have a cyano group attached to a modified prolyl residue. The instant claims recite compounds wherein a cyano group is not listed among the substituents of the corresponding prolyl residue. Therefore, Applicants assert that an anticipation rejection cannot be sustained.

Moreover, Applicants have added an element to claims 38-40 which specifies that the composition is administered in a single daily dosage. Applicants point out that Villihauer does not teach or suggest this element. Quite on the contrary, Applicants have included a reference by Villhauer which discloses that Villhauer's compounds are intended for administering multiple dosages a day. (See, e.g., Appendix 3, page 5, first full paragraph).

In view of the arguments presented above, Applicants respectfully request reconsideration and removal of the rejection to claims 38-40 and claims dependent thereon.

14. Claims 38-41, 44-52, 54-57, 59, 60, 63 and 66 are rejected under 35 U.S.C. 102(b) as being anticipated by inventor Demuth's German Patent 19616486 (hereinafter referred to as the '486 patent). Applicants traverse the rejection to the extent that it is maintained over the amendments entered.

The instant claims, as amended, are directed to uses of DPIV inhibitors to modify glucose metabolism in a glucose intolerant animal. Applicants assert that the '486 patent does not provide an enabled disclosure which teaches modifying glucose metabolism in a glucose intolerant animal. Applicants have not found any examples in the '486 patent

showing the efficacy of the compounds cited therein for modifying glucose metabolism in an glucose intolerant animal. In contrast, Applicants have demonstrated the efficacy of the instantly claimed compounds by treating animals with glucose intolerance. Thus the '486 patent does not disclose every limitation of the pending claims, and therefore does not anticipate the claims.

Applicants further point out that there is a critical difference between the compounds disclosed Demuth in the '486 patent, and the compounds of the instant claims. Demuth's compounds, i.e., alanyl pyrolidide, isoleucyl thiazolidide, N-valylproly, and O-benzoyl hydroxyl amine, are compounds as Demuth et al. put it "that are rapidly reversible inhibitors that are of a type that are quickly cleared from circulation." (See Demuth et al. Appendix II, at page 140, left column, line 16) In fact, Demuth et al., in considering DPIV inhibitors optimal for design of antidiabetic agents, specifically endorse these types of inhibitors as being the most appropriate. Applicant's compounds, however, are of the type that form covalent adducts with DP IV, and thus are apparently not of the type taught by Demuth would be optimal antidiabetic agents. Applicants submit that such differences between Demuth's compounds and the instantly claimed compounds are further evidence that the Demuth reference does not anticipate the present claims.

Moreover, Applicants have added an element to claims 38-40 which specifies that the composition is administered in a single daily dosage. Applicants point out that Demuth does not teach or suggest this element.

In view of the arguments presented above, Applicants respectfully request reconsideration and removal of the rejection to claims 38-40 and claims dependent thereon.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants submit that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Should an extension of time be required, Applicants hereby petition for same and request that the extension fee and any

other fee required for timely consideration of this submission be charged to **Deposit Account No. 18-1945.**

Respectfully Submitted,

Date: 9/23/03

David P. Halstead, J.D., Ph.D.
Reg. No. 44,735

Customer No: 28120
Docketing Specialist
Ropes & Gray LLP
One International Place
Boston, MA 02110
Phone: 617-951-7000
Fax: 617-951-7050

Appendix 1

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Ovid Technologies, Inc. Email Service
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Search for: from 3 [2 and receptor.mp. [mp=title, abstract, cas
registry/ec number word, mesh subject heading]] keep 10
Citations: 1

Citation <1>
Unique Identifier
  9381748
Medline Identifier
  97442845
Authors
  Gallwitz B.  Schmidt WE.
Title
  [GLP-1 receptor gen "knock out" causes glucose intolerance, but no
alterations of eating behavior]. [German] Source
  Zeitschrift fur Gastroenterologie.  35(8):655-8, 1997 Aug.
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Appendix II

ELSEVIER

Regulatory Peptides 96 (2001) 133–141

REGULATORY PEPTIDES

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Metabolism of glucagon by dipeptidyl peptidase IV (CD26)

John A. Pospisilik[c,1], Simon A. Hinke[c,1], Raymond A. Pederson[c], Torsten Hoffmann[a], Fred Rosche[a], Dagmar Schlenzig[a], Konrad Glund[a], Ulrich Heiser[a], Christopher H.S. McIntosh[c], Hans-Ulrich Demuth[a,b,*]

[a]*Probiodrug Research, Biocenter, Weinbergweg 22, D-06120 Halle (Saale), Germany*
[b]*Department of Drug Biochemistry, Hans-Knoell Institute of Natural Product Research, Biocenter, Weinbergweg 22, D-06120 Halle (Saale), Germany*
[c]*Department of Physiology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3*

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Abstract

Glucagon is a 29-amino acid polypeptide released from pancreatic islet α-cells that acts to maintain euglycemia by stimulating hepatic glycogenolysis and gluconeogenesis. Despite its importance, there remains controversy about the mechanisms responsible for glucagon clearance in the body. In the current study, enzymatic metabolism of glucagon was assessed using sensitive mass spectrometric techniques to identify the molecular products. Incubation of glucagon with purified porcine dipeptidyl peptidase IV (DP IV) yielded sequential production of glucagon$_{3-29}$ and glucagon$_{5-29}$. In human serum, degradation to glucagon$_{3-29}$ was rapidly followed by N-terminal cyclization of glucagon, preventing further DP IV-mediated hydrolysis. Bioassay of glucagon, following incubation with purified DP IV or normal rat serum demonstrated a significant loss of hyperglycemic activity, while a similar incubation in DP IV-deficient rat serum did not show any loss of glucagon bioactivity. Degradation, monitored by mass spectrometry and bioassay, was blocked by the specific DP IV inhibitor, isoleucyl thiazolidine. These results identify DP IV as a primary enzyme involved in the degradation and inactivation of glucagon. These findings have important implications for the determination of glucagon levels in human plasma.

Keywords: Rodent; Peptide inactivation; Incretins; Glucose; GIP; GLP-1; MALDI-TOF mass spectrometry

1. Introduction

Glucose homeostasis is maintained in large part by a balance between the anabolic actions of insulin and the catabolic actions of the glucose counter-regulatory hormone, glucagon. Altered secretion and responsiveness of glucagon is a hallmark of diabetes mellitus: hyperglucagonemia is observed in types 1 and 2 diabetes, and there is a diminished glucagon response to hypoglycemia in type 1 diabetes [1].

Glucagon shares sequence identity with the gut hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Together, GIP and GLP-1 comprise the incretin component of the entero-insular axis and account for approximately 50% of nutrient-stimulated insulin secretion [2,3]. In recent years, it has been demonstrated by ourselves and others that the circulating serine protease dipeptidyl peptidase IV (DP IV; EC 3.4.14.5; CD26) rapidly cleaves the N-terminal dipeptides from these incretins [4–12] both in vitro and in vivo, yielding truncated, biologically inactive GIP$_{3-42}$ and GLP-1$_{9-36amide}$. The physiological role played by DP IV in the regulation of incretin activity, and thus in the regulation of blood glucose, was recently established by studies showing enhanced insulin secretion and improved glucose tolerance resulting from the administration of specific DP IV inhibitors [8,10]. Rats lacking the enzyme DP IV were

*Corresponding author. Tel.: +49-345-555-9900; fax: +49-345-555-9901.

E-mail address: hans-ulrich.demuth@probiodrug.de (H.-U. Demuth).

[1]John A. Pospisilik and Simon A. Hinke contributed equally to this work.

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PII: S0167-0115(00)00170-1

shown by us to have normal glucose tolerance, despite having longer acting incretins, leading to the hypothesis of compensatory mechanisms, possibly involving glucagon, that were activated to prevent hypoglycemia [9].

The circulating half-life of glucagon has been reported to be in the range of 5 to 6 min [13,14]. The literature regarding degradation of glucagon by the liver is controversial. It has been reported that hepatic metabolism of glucagon is mediated by the cytosolic enzyme dipeptidyl peptidase I (DP I; EC 3.4.14.1; cathepsin C) [15–17]. However, in reviewing the contribution of the liver to glucagon degradation, Holst concluded that in dogs, as well as humans, there is low hepatic extraction of glucagon [18] This conclusion is supported by findings that glucagon was not degraded by passage through the perfused rat liver [19]. Consensus exists that the kidney plays a major role in the metabolic clearance and degradation of glucagon [18]. Studies by several groups [20–22] indicated that after glomerular filtration in the kidney, glucagon is hydrolyzed by brush border enzymes in the proximal tubule. Relevant to this finding and the current study is the presence of high concentrations of DP IV in the renal tubular brush border [23,24].

The enzyme DP IV is substrate-specific, preferentially cleaving dipeptides from the N-terminus of peptides with a proline or alanine in the penultimate (P_1) position [25]. Recently, in a study of DP IV utilizing low-molecular-weight peptide substrates, the substrate specificity was extended to include peptides containing P_1 Hyp, Ser, Gly, Val, or Leu residues [25–27]. Thus glucagon, with a P_1 serine, became a potential candidate for DP IV-mediated degradation.

The inclusion of glucagon as a potential DP IV substrate, and the absolute requirement for an intact N-terminus of glucagon for biological activity, therefore provided the rationale for studying glucagon metabolism by DP IV [28,29]. In the current study, products of in vitro glucagon hydrolysis were characterized using matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI–TOF MS). In vivo studies demonstrated that N-terminal truncation of the peptide by DP IV renders it biologically inactive. In a parallel study, we have shown that N-terminally modified glucagon analogs (truncated, cyclized and/or P_1/P_1 substituted) have reduced binding affinity and efficacy, when tested on cells transfected with the human glucagon receptor [30]. These findings are consistent with a role of DP IV in glucagon degradation.

2. Materials and methods

2.1. Peptide synthesis and purification

Glucagon and synthetic fragments were synthesized using an automated peptide synthesizer SYMPHONY (RAININ), applying an Fmoc-protection protocol. All reagents for peptide synthesis were analytical grade and obtained from Novabiochem (Schwalbach, Germany), Roth (Karlsruhe, Germany) or J.T. Baker (Griesheim, Germany). Products were purified by HPLC, and MALDI–TOF mass spectrometry was used to confirm peptide identity and purity. Synthetic peptides used in this study are described in more detail elsewhere [30].

2.2. DP IV and inhibitors

Dipeptidyl peptidase IV from pig kidney was purified according to the method described by Wolf et al. [31]. The specific activity was measured spectrophotometrically ($\lambda = 390$ nm) using Gly-Pro-4-nitroanilide as a chromogenic substrate [7,8,12]. Activity of DP IV used in these studies was 31 units/mg protein; one unit of DP IV activity is defined as the rate of appearance (μM/min) of yellow product, 4-nitroaniline, from 400 μM substrate in HEPES buffer (40 mM, pH 7.6, 30°C). Human serum was pooled from healthy subjects, and diluted to 20% in Tris buffer (0.1 mM, pH 7.6) for use in experiments. The highly specific, reversible, competitive transition-state analogue inhibitor of DP IV, isoleucyl thiazolidine (Ile-Thia), synthesized in our laboratory, has been previously described in the literature [8,25]. Generally, analysis of degradation was measured qualitatively and quantitatively using capillary zone electrophoresis or MALDI–TOF MS. Glucagon (2.5 μM) was incubated in either 2.5 mU/ml of purified porcine DP IV or 20% human serum in Tricine buffer with or without Ile-Thia (20 μM) as previously described [7]. Samples were removed at various time points and analyzed.

2.3. Capillary zone electrophoresis (CE)

CE analysis of DP IV-mediated glucagon degradation was carried out on a P/ACE 2050 system (Beckman Coulter GmbH, München, Germany) using a fused-silica capillary (diameter: 50 μm, effective length: 20 cm). Samples were injected with positive pressure for 8 s. A constant voltage (14 kV) was used to separate peptides, using a sodium phosphate buffer (0.1 M, pH 2.5). Absorption of the peptides was monitored at a wavelength of 200 nm. Calculations of enzyme kinetics were performed according to established methods [7].

2.4. Matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI–TOF MS)

MALDI–TOF MS analysis of DP IV-catalyzed peptide proteolysis using a Hewlett–Packard G2025 mass spectrometer has been previously documented [7]. Briefly, at various time points, a 1:1 mixture of analyte and matrix solution (2′,6′-dihydroxyacetophenone with diammonium hydrogen citrate) were combined and 1 μl was transferred to the sample tip. The analyte/matrix complex was evapo-

rated immediately in a vacuum chamber (sample preparation accessory HP G2024A), in order to obtain a homogeneous crystallization. Each mass spectrum represents the cumulative *m/z* signal for 250 single laser shots (laser power 1.0–2.5 μJ).

2.5. Bioassay

In vivo testing of glucagon and N-terminally truncated fragments was carried out using a bioassay monitoring blood glucose concentration. Degradation of glucagon by purified pig kidney DP IV, normal Wistar or Fischer 344 rat plasma, or DP IV negative Fischer 344 rat plasma, with or without DP IV inhibitor (Ile-Thia, 50 μM) was assessed. Degradation of glucagon by purified DP IV was assessed by incubation with 0.31 units of DP IV in 1.0 ml of phosphate-buffered saline (PBS, pH 7.4) at 37°C for 3.25 h, followed by subcutaneous (SC) injection into conscious unrestrained male Wistar rats (225–275 g). Similarly, glucagon was incubated with normal male (Wistar or Fischer) rat serum or DP IV negative rat serum (1.0 ml) under the same conditions (37°C, 3.25 h), prior to SC injection. Concurrent experiments using 50 (micro)M Ile-Thia under the same conditions were assayed by IV injection into anesthetized male Wistar rats (65 mg/kg sodium pentobarbital; Somnotol, MTC Pharmaceuticals, Cambridge, Ontario, Canada). DP IV negative rats were obtained from a colony maintained in the Department of Physiology, UBC, Vancouver, Canada [9]. Control vehicle (saline or plasma) injections were also given in all experimental protocols. Bioactivity of synthetic N-terminally truncated peptides dissolved in saline was tested using analogue doses at ten-fold higher concentration than for native glucagon (doses: 7.1 nmol/kg glucagon$_{1\ 29}$, and 71 nmol/kg for glucagon$_{3\ 29}$, pyroglutamyl-glucagon$_{3\ 29}$ {[pGlu^3]glucagon$_{3\ 29}$} and glucagon$_{5\ 29}$). Whole blood glucose concentration was measured using tail bleeds and a SureStep® glucose analyser (LifeScan Canada, Burnaby, B.C., Canada). Animal work was performed in accordance with the guidelines set out by Principles of Animal Laboratory Care (NIH publication No. 85-23, revised 1985).

2.6. Data analysis

Data are presented as mean±standard error of the mean (S.E.M.), with the number of experiments shown in the figure legends. Statistical significance was set at the 5% level, and assessed using analysis of variance (ANOVA) and Dunnett's Multiple Comparison Test or the Newmann–Keuls Test as post hoc tests of significance where appropriate. Data analysis was done using the Prism software package (GRAPHPAD, San Diego, CA, USA). Blood glucose data are presented as fold-basal activity.

3. Results

3.1. DP IV and serum degradation products of glucagon

Molecular species resulting from incubation of glucagon$_{1\ 29}$ (3483.8 Da) in purified porcine DP IV were identified by MALDI–TOF mass spectrometry. DP IV-catalyzed loss of the N-terminal dipeptide of glucagon (His^1-Ser^2) was indicated by the appearance of glucagon$_{3\ 29}$ (3259.6 Da) in the analyte (Fig. 1). A second degradation product corresponding to glucagon$_{5\ 29}$ (3074.6 Da) appeared over a longer time course indicating subsequent N-terminal cleavage of Gln^3-Gly^4 from glucagon$_{3\ 29}$. Both degradation steps were completely blocked by the addition of 20 μM isoleucyl thiazolidine (Ile-Thia), a non-hydrolyzable, reversible DP IV-specific inhibitor (data not shown).

Incubation of glucagon in 20% human serum showed a single step degradation to a single molecular species with the molecular weight of 3242.5 Da (Fig. 2A), inclusion of 20 μM Ile-Thia in the incubation prevented formation of this product (Fig. 2B) identifying DP IV as the protease responsible. This product was 17 Da less than the mass of

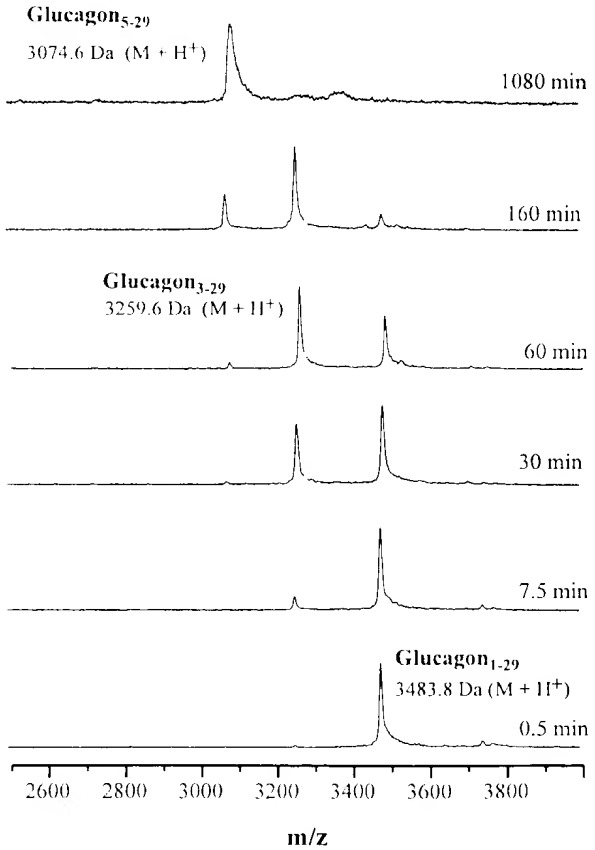

Fig. 1. Degradation of glucagon by purified porcine DP IV monitored by MALDI TOF mass spectrometry. Refer to Section 2 for specific methods.

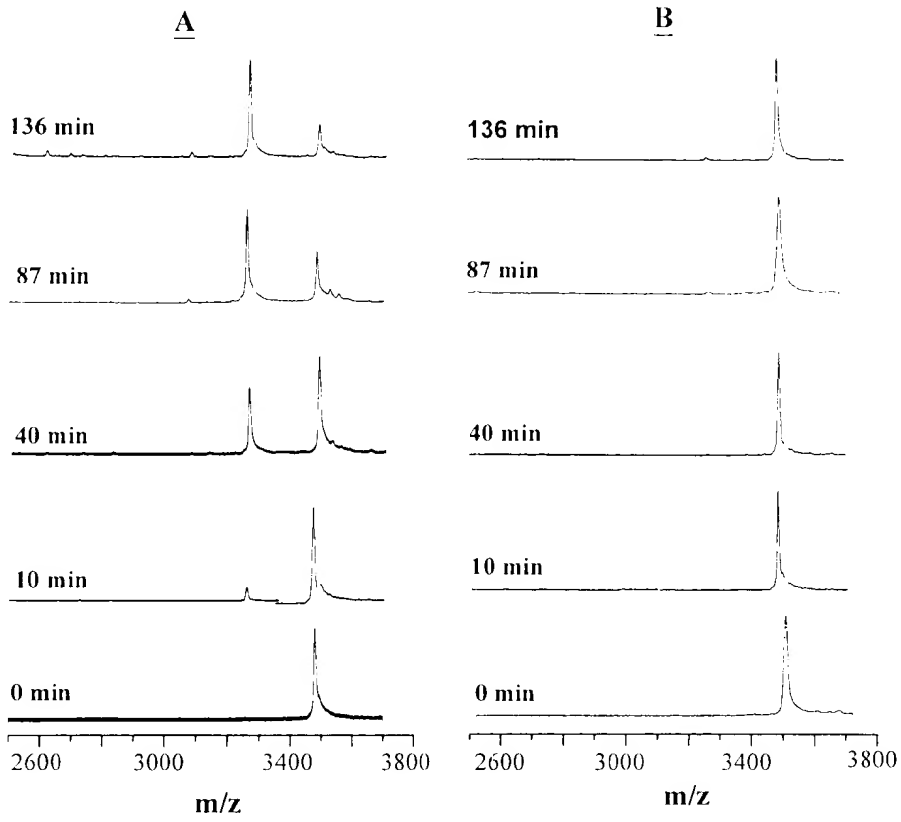

Fig. 2. Degradation of glucagon by 20% human serum monitored by MALDI–TOF mass spectrometry in the absence (A) and presence (B) of the DP IV inhibitor, Ile-Thia. Refer to Section 2 for specific methods.

the expected product, glucagon$_{3\text{-}29}$ (3256.7 Da); comparison with synthetic glucagon$_{3\text{-}29}$ by MALDI–TOF MS revealed two distinct molecular species (Fig. 3). Incubation of glucagon$_{3\text{-}29}$ in 20% human serum for 2 h also gave rise to this slightly smaller molecular species (Fig. 4). Glucagon$_{3\text{-}29}$-17 Da from human serum incubations was

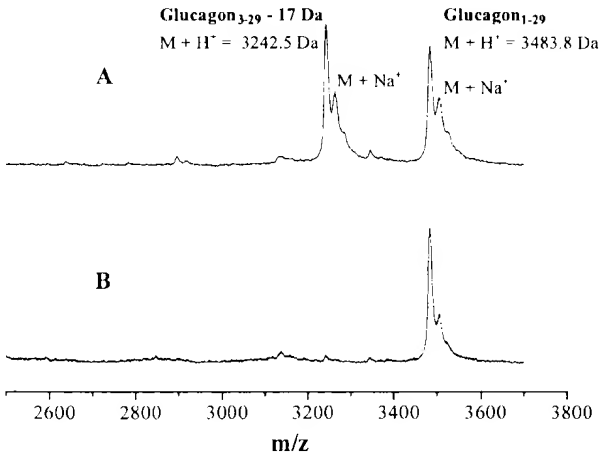

Fig. 3. Degradation of glucagon by 20% human serum monitored by MALDI–TOF mass spectrometry in the absence (A) and presence (B) of the DP IV inhibitor, Ile-Thia. Refer to Section 2 for specific methods.

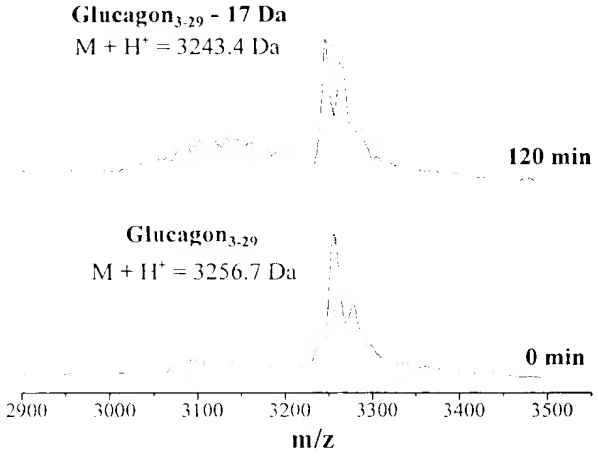

Fig. 4. Formation of a cyclized derivative of glucagon$_{3\text{-}29}$, measured by MALDI–TOF mass spectrometry. Glucagon$_{3\text{-}29}$ is converted by human serum to a peptide 17 Da smaller, the equivalent mass of an amide group.

indistinguishable from synthetic [pGlu^3]glucagon$_{3\text{-}29}$ by MALDI–TOF MS, HPLC and capillary electrophoresis (data not shown).

Moreover, incubation of both peptides, glucagon$_{1\text{-}29}$ and glucagon$_{3\text{-}29}$, in 20% human serum and in the presence of 10 μM of the highly specific, irreversible inhibitor of DP I,

N-(H-Gly-Phe-), *O*-benzoyl hydroxylamine, did not alter the observed glucagon degradation pattern (details to be published elsewhere).

3.2. Kinetic analysis of DP IV-mediated glucagon degradation

Kinetic characterization of the N-terminal degradation of glucagon$_{1-29}$ to glucagon$_{3-29}$ resulting from incubation of glucagon with purified porcine kidney DP IV was performed by capillary zone electrophoresis (Fig. 5). Molecular species were identified which corresponded to those observed by MALDI–TOF MS and time course studies were performed allowing calculation of enzyme kinetic parameters, K_m (Michaelis constant), k_{cat} (maximum velocity), and k_{cat}/K_m (specificity constant or second order rate constant). These results are summarized in Table 1 together with those for several other peptide substrates of DP IV taken from Mentlein et al. [5,32] Notably, the

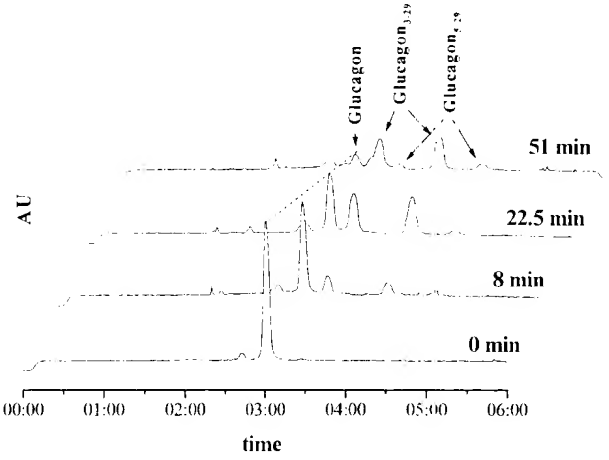

Fig. 5. Degradation of glucagon by purified porcine DP IV measured with capillary zone electrophoresis. Kinetic parameters of results can be found in Table 1. See Section 2 for specific methods. Clear sequential degradation to glucagon$_{3-29}$ and glucagon$_{5-29}$ is shown. Note that the latter fragments appear as double peaks, an effect likely due to the resolution of *cis*/*trans* isoforms of the truncated molecules. The same can be shown for purified synthetic glucagon$_{3-29}$.

Table 1
Kinetic parameters of DP IV-catalyzed peptide hydrolysis, determined by chromatographic methods

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} s^{-1}$)
Glucagon$_{1-29}$[a]	3.9	0.74	194
GIP[b]	34	7.6	220
GLP-1$_{7-36}$[b]	4.5	1.9	430
PYY[c]	28	52	1900
NPY[c]	20	22	12 000

[a] Parameters estimated by capillary zone electrophoresis (Materials and methods).
[b] Data from Ref. [5] determined by HPLC.
[c] Data from Ref. [32] determined by mass spectrometry.

enzymatic cleavage of the first dipeptide (His^1-Ser^2) occurred at a more rapid rate than did the removal of the second (Gln^3-Gly^4).

3.3. Bioassay analysis of DP IV degradation

Degradation of glucagon in purified DP IV and rat plasma was investigated using a bioassay for the hyperglycemic effect of glucagon. Incubation of glucagon$_{1-29}$ in purified DP IV or normal male Wistar rat plasma resulted in a significant loss of its hyperglycemic activity, when injected into either conscious or anesthetized Wistar rats (Figs. 6 and 7 respectively). Conversely, 50 μM Ile-Thia completely blocked the loss of bioactivity under the same conditions (Fig. 7). Comparison of the bioactivity of glucagon incubated in DP IV-negative or Fischer rat plasma shows that bioactivity is lost when incubated in Fischer plasma, but is only slightly reduced after incubation in DP IV-negative plasma relative to glucagon in saline (Fig. 6B). Bioassay of synthetic glucagon$_{3-29}$, [pGlu^3]glucagon$_{3-29}$, and glucagon$_{5-29}$ indicated that these peptides had negligible hyperglycemic activity at ten-fold higher doses than native glucagon (Fig. 6C).

4. Discussion

As discussed in the Introduction, there has been considerable controversy in the literature regarding the specific tissues and enzymes responsible for the degradation of glucagon. The current study specifically examined the possible involvement of the ubiquitous enzyme, dipeptidyl peptidase IV, in the metabolism of glucagon. Assessment of glucagon degradation products by capillary zone electrophoresis and MALDI–TOF spectrometry has identified the specific molecular species produced from glucagon metabolism both in human serum and by purified porcine DP IV. The data provide strong evidence that DP IV is a primary candidate for enzymatic degradation of glucagon.

Previous reports on degradation of glucagon by DP I were not confirmed. Similar to DP IV, DP I removes amino-terminal dipeptides, however, DP I is a promiscuous enzyme with a much broader substrate specificity [15–17,33]. In contrast to DP IV, DP I acts intracellularly, on the internal surface of lysosomal membranes, while DP IV is present as a membrane-bound ectoenzyme and a freely circulating enzyme [17,24,25]. Inhibition of DP I activity by specific inhibitors had no effect on the degradation of glucagon in human serum, indicating that DP I was not the enzyme responsible for glucagon degradation observed in serum. In contrast, inhibition of DP IV by Ile-Thia completely blocked serum mediated glucagon degradation (Fig. 2). Further support for the role of DP IV in glucagon metabolism was found using purified pig kidney DP IV, which gave rise to the expected N-terminally truncated peptide, glucagon$_{3-29}$ in addition to an N-terminally

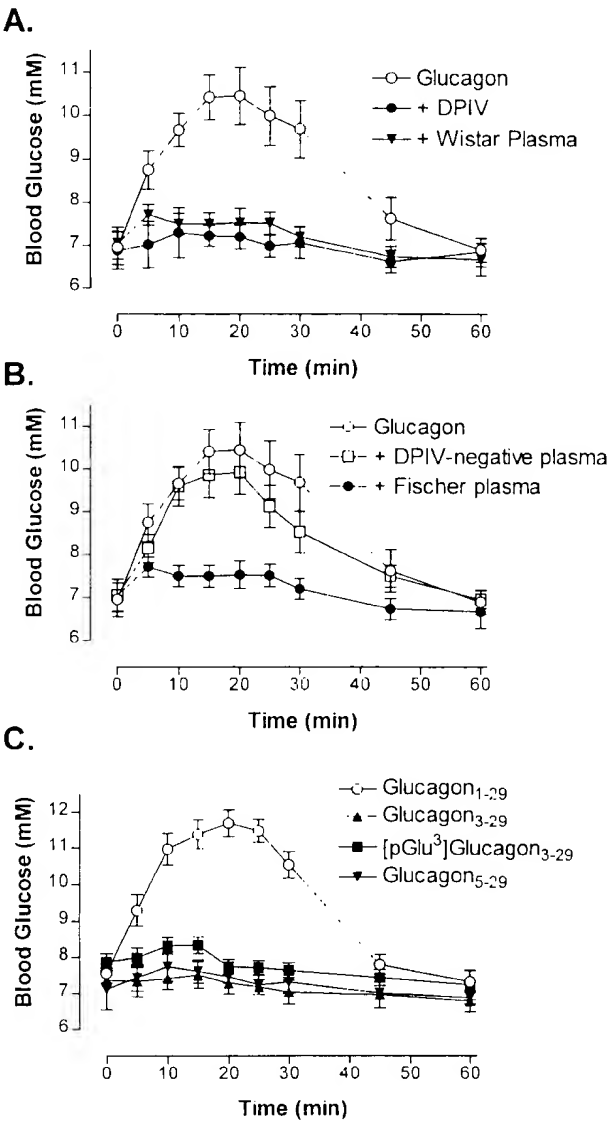

Fig. 6. Degradation and bioactivity of glucagon and N-terminally truncated analogues in vivo. (A and B) Glucagon (7.1 nmol/kg) was incubated in 1 ml saline, 0.31 mU purified DP IV, or rat plasma (37°C, 3.25 h) prior to subcutaneous injection into unrestrained, conscious male Wistar rats. (C) Synthetic glucagon analogues (71 nmol/kg) corresponding to DP IV degradation products were similarly bioassayed. Blood glucose was measured using a SureStep blood glucose analyzer. N = 6 for each group.

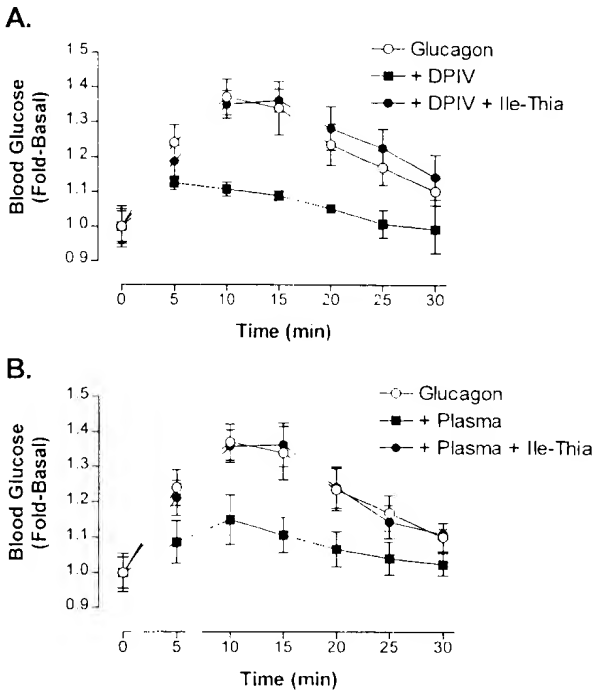

Fig. 7. Inhibition of DP IV and plasma degradation of glucagon by Ile-Thia monitored by bioassay. (A) Glucagon (7.1 nmol/kg) was incubated in saline with purified DP IV in the presence or absence of Ile-Thia (37°C, 3.25 h), prior to intravenous injection into anesthetized male Wistar rats. (B) Glucagon was similarly incubated in normal male Wistar rat serum with or without Ile-Thia, prior to IV injection. N = 4 for each group.

truncated product, glucagon$_{5-29}$, the result of a second N-terminal dipeptide cleavage step (Fig. 1). During preparation of this manuscript, another research group reported a similar sequential degradation of macrophage-derived chemokine (MDC) by DP IV [34]. Together with this report we have now shown that all known members of the GRF-superfamily of polypeptides are substrates for DP IV hydrolysis (data to be published elsewhere).

When examining degradation of glucagon by purified DP IV, the sequential loss of dipeptides resulted in the expected molecular weights for the truncated N-terminal fragments glucagon$_{3-29}$ and glucagon$_{5-29}$ (Fig. 1). However, incubation of glucagon$_{1-29}$ in serum consistently gave rise to a peptide product 17 Da less than the expected weight for glucagon$_{3-29}$: glucagon$_{3-29}$ − 17 Da (Figs. 2 and 3). Incubation of glucagon$_{3-29}$ in human serum also showed a rapid conversion to glucagon$_{3-29}$ − 17 Da (Fig. 4). Hence it appears that in serum, DP IV first cleaves the N-terminal dipeptide from glucagon, and a serum enzyme then catalyzes the cyclization of the amino terminal glutamine residue (Gln^3), resulting in the loss of an NH_3 group (17 Da). Transglutaminases are well documented serum enzymes which cause the formation of new γ-carbox-amide bonds between the γ-carboxyl groups of glutamine and primary amines [35]. No intermediate glucagon$_{3-29}$ was observed in human serum, therefore the DP IV-catalyzed N-terminal cleavage reaction appears to be rate determining. Furthermore, as glucagon$_{5-29}$ was not observed, it appears that the cyclization step occurs much more rapidly than the second DP IV-mediated truncation step (cleavage of glucagon$_{3-29}$ to glucagon$_{5-29}$). In order to render the peptide resistant to further degradation by DP IV, the cyclization event must involve either the α-amine or γ-carbox-amide of Gln^3. Products with the correct molecular mass could include pyroglutamyl-glucagon$_{3-29}$

([pGlu^3]glucagon$_{3-29}$), cyclo$^{1-12}$-glucagon$_{3-29}$, cyclo$^{1-20}$-glucagon$_{3-29}$ or cyclo$^{1-24}$-glucagon$_{3-29}$. When used as a standard for HPLC, MALDI–TOF MS, and CE, synthetic [pGlu^3]glucagon$_{3-29}$ could not be discriminated from the serum-catalyzed glucagon$_{3-29}$ − 17 Da. Hence, it is likely that this is the product formed in serum, however, the possibility of other cyclic derivatives formed in vivo needs further investigation. The characteristics of this cyclization are consistent with the activity of the serum enzyme, glutaminyl cyclase [36].

In vitro enzymology studies identify DP IV as the primary enzyme involved in the metabolism of glucagon in the circulation. Further studies were carried out using a bioassay in rats to substantiate in vitro results and determine the in vivo consequences of N-terminal truncation and cyclization. Incubation of glucagon in normal rat plasma or purified porcine DP IV resulted in a complete loss of the hyperglycemic response to glucagon in vivo (Fig. 6A). Consistent with the in vitro assays, use of Ile-Thia prevented the loss of bioactivity of glucagon incubated in serum or DP IV (Fig. 7). Further evidence supporting DP IV-mediated metabolism of glucagon was provided by incubation of glucagon in DP IV-negative rat plasma. As expected, glucagon incubated in DP IV-negative plasma retained bioactivity, in contrast to control Fischer rat plasma (Fig. 6B). Concurrently with this study, the binding and activation characteristics of the truncated glucagon fragments were assessed in vitro using cells transfected with the human glucagon receptor (33). All three peptides (glucagon$_{3-29}$, [pGlu^3]glucagon$_{3-29}$, and glucagon$_{5-29}$) showed weak agonist activity with five- to 18-fold lower binding affinities than the native glucagon molecule. The current data are in agreement with these findings.

In addressing the physiological relevance of DP IV-mediated glucagon degradation in vivo, it is important to consider several points: first, the majority of the DP IV activity in the circulation is membrane bound, endothelial DP IV (at least one order of magnitude higher than plasma DP IV) [37], and therefore, the majority of the conversion of glucagon to its truncated form, in vivo, likely occurs at the blood vessel wall rather than in the plasma matrix. Among peptide substrates, glucagon exhibits the highest affinity for DP IV tested so far [25,38], in addition to a second-order rate constant on par with those of the incretins GIP and GLP-1 (Table 1). Taken together with the fact that the circulating concentration ranges of the three hormones are similar and the physiological impact of DP IV activity on incretin action, it is likely that DP IV plays a significant role in the clearance of active glucagon from the circulation. This is supported by our parallel characterization of D-Ser^2-glucagon, a synthetic DP IV-resistant glucagon analog with receptor binding and activation characteristics equivalent to those of native glucagon. Using the same bioassay as outlined above, D-Ser^2-glucagon was shown to elicit a prolonged and increased hyperglycemic response when compared to native glucagon [30].

Comparison of the second-order rate constants of the three glucoregulatory peptides discussed so far, with those of previously characterized bioactive peptide DP IV substrates, now becomes very fitting. That is to say, glucagon, GIP, and GLP-1 (k_{cat}/K_m ~200–400 mM^{-1} s^{-1}), which must move through the circulation to their target tissues are afforded a relative DP IV-resistance over, for example, the locally acting neuropeptide DP IV-substrates NPY and PYY, which are degraded much more rapidly (k_{cat}/K_m = 2000 and 12 000 mM^{-1} s^{-1}, respectively; Table 1; [32]). Potential overstimulation of local paracrine pathways (e.g. glucagon stimulation of insulin secretion) by the more stable peptide hormones is likely avoided by co-release of DP IV and localization of DP IV to the target tissues. A recent finding of Grondin and colleagues, the localization of DP IV to the pancreatic α-cell secretory granule [39] gives support to this idea, as well as giving rise to the possibility of local effects of the truncated peptide fragments (dipeptides and N-terminally truncated).

As the glucagon degradation products (glucagon$_{3-29}$ and [pGlu$_3$]glucagon$_{3-29}$, or other possible cyclized derivatives) are found in human serum, measurements of glucagon immunoreactivity by 'side-viewing' or C-terminally directed antibodies [40] would likely cross react with these peptides. Studies using antibodies for the measurement of glucagon may therefore be misleading and this is likely the reason that glucagon was previously reported to be stable in plasma [14,41]. Frandsen et al. found that glucagon$_{5-29}$ cross-reacted with at least two antisera tested (K5563 and K4023) [42]. Many such antibodies probably also cross-react with glucagon$_{3-29}$ and [pGlu^3]glucagon$_{3-29}$ (or other cyclized derivatives). GLP-1$_{7-36}$ is degraded to GLP-1$_{9-36}$ by DP IV [4]. An N-terminal antibody to GLP-1$_{9-36}$ exists, allowing determination of circulating levels of intact GLP-1, determined by a subtractive method. The circulating concentration of GLP-1$_{9-36}$ was found to be approximately ten-times higher than the full length hormone [43]. Work is underway to develop assay techniques (both ELISA and radioimmunoassay) specific for the active N-terminally intact, as well as the cyclized and truncated, glucagon forms. Such assays, in addition to studies with larger animal models, are necessary for definitive characterization of the conversion of glucagon$_{1-29}$ to [pGlu^3]glucagon$_{3-29}$ in vivo.

Considerable attention has been given to the development of DP IV resistant GLP-1 [44–47] and GIP [48,49] analogues, or inhibition of DP IV [8,10,12] to improve glucose tolerance in type 2 diabetic patients. Generation of synthetic incretin analogs with increased resistance to DP IV has led to peptides with increased circulating half-lives and enhanced antidiabetic effects owing to prolonged insulinotropic activity [44,47,49]. Similarly, inhibition of DP IV with the specific inhibitors, Ile-Thia and valyl-pyrrolidine, improved glucose tolerance in animal models,

likely through enhancing the activity of endogenous incretins [8,10,12]. Given that DP IV is also responsible for degrading glucagon, the improved glucose tolerance seems counter-intuitive, as inhibition of DP IV would also enhance the activity of endogenous glucagon. However, postprandially, both the stimulation, and thus the rate of endogenous glucagon secretion, are at a minimum, while those of the incretins are a maximum. Thus any enhancement of glucagon activity is likely masked by a much greater relative enhancement of incretin activity. Also, remaining intact negative feedback loops for glucagon continue to be active (in particular the glucagonostatic action of GLP-1 is likely enhanced), while the secretion stimulus for the incretins continues. It follows then, that when considering the design of DP IV inhibitors as antidiabetic agents, rapidly reversible inhibitors that are quickly cleared from the circulation are most desirable. Considering the data presented so far, it is likely that the physiological consequences of DP IV inhibition shift in tandem with this transition in regulatory control, from an insulin enhancing effect (incretin stabilizing) to a glucagon enhancing one (glucagon stabilizing) due to the relative changes in concentration of these circulating hormones in the postprandial state. When taken together with the fact that approximately two thirds of the 24 h cycle is spent in the fasting state, primarily under the control of glucagon, it is obvious that slow-binding, irreversible or stable inhibitors which sustain DP IV inhibition into the fasting state, would likely become counterproductive as antidiabetic drugs.

In summary, this study of glucagon metabolism has shown that DP IV is a primary enzyme involved in glucagon degradation. Purified porcine kidney DP IV was shown to sequentially degrade glucagon$_{1\ 29}$ to glucagon$_{3\ 29}$ and then glucagon$_{5\ 29}$. In human serum, cyclization of the truncated N-terminus prevented DP IV-mediated hydrolysis beyond glucagon$_{3\ 29}$. In vitro and in vivo, the amino-terminal truncation of glucagon was specifically blocked by the DP IV inhibitor Ile-Thia. Bioassay of synthetic fragments corresponding to DP IV degradation products revealed that truncated fragments possessed no hyperglycemic activity in vivo, leaving the physiological role of these peptides unknown. These results comprise a major step in the characterization of glucagon metabolism and thus contribute towards our understanding of diseases involving abnormal glucose counter-regulation.

Acknowledgements

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Leading Article

The role of glucagon-like peptide-1 and its analogues in the treatment of diabetes

Jens Juul Holst
Department of Medical Physiology, University of Copenhagen, The Panum Institute, Copenhagen, Denmark (holst@mfi.ku.dk)

Type 2 diabetes mellitus and the incretin hormones

The incretin hormones are intestinal hormones that augment insulin secretion in response to nutrients in addition to the effect of the nutrients alone [1, 2]. The incretin effect is often quantified by comparing the insulin response to oral glucose and to an intravenous glucose infusion by precisely matching the glucose response to the oral load. In humans, two related gastrointestinal peptide hormones are thought to be responsible for the incretin effect, namely glucose-dependent insulinotropic polypeptide (GIP, previously called gastric inhibitory polypeptide) and glucagon-like peptide-1 (GLP-1). Both are secreted in response to meal ingestion [3, 4], and mimicry experiments [5, 6] have shown that both peptides are powerful potentiators of glucose-induced insulin secretion in humans and that they have additive effects on insulin secretion. Their importance in the regulation of glucose metabolism is also evident from experiments involving deletion of the genes encoding their receptors. Knockout of the GIP receptor is associated with significant glucose intolerance [7]: mice without the GLP-1 receptor are glucose-intolerant and may exhibit fasting hyperglycaemia [8].

One would therefore assume that defects in the incretin effect in humans would be associated with glucose intolerance. Indeed, in experiments with isoglycaemic oral and intravenous glucose administration in patients with type 2 diabetes mellitus, a severely reduced incretin effect was demonstrated [9]. At present it is not known to what extent the incretin defect in type 2 diabetes contributes to the metabolic dysregulation, but there is little doubt that the defect contributes to the impaired insulin secretion that characterizes the disease.

What is the nature of the incretin defect? Careful studies of the secretion of GLP-1 and GIP in patients with type 2 diabetes have shown that meal-induced GIP secretion is normal or near-normal, whereas GLP-1 secretion is significantly impaired [10]. Impaired secretion of GLP-1 is thought to be secondary to diabetes because, in identical twins discordant for diabetes, impaired secretion has only been observed in the diabetic twin [11].

How effective are the incretin hormones in patients with type 2 diabetes? Careful clamp studies have shown that whereas near-normal insulin responses were observed after infusion of GLP-1, the effect of GIP was almost abolished in the patients and greatly reduced in comparison with that of matched control subjects [12]. Similar observations have been made by others [13].

Biological effects of GLP-1

It may therefore be concluded that there is a severely impaired incretin effect in type 2 diabetes and that the defect results mainly from an impaired secretion of GLP-1 plus an impaired effect of GIP. Although clearly transmitted through separate and highly specific receptors on the b-cells, the two hormones are thought to activate the same intracellular machinery, both involving cAMP accumulation [14]. In order to remedy the incretin defect in type 2 diabetes it would seem logical to provide these patients with GLP-1 in order to substitute both the deficient secretion of GLP-1 and the deficient effect of GIP. Indeed, intravenous infusions of GLP-1 have been shown to be able to completely normalize the hyperglycaemia of type 2 diabetic patients regardless of the severity of disease [15, 16]. Furthermore, apart from its insulinotropic actions, GLP-1 has an additional number of effects, all of which are highly desirable in the context of type 2 diabetes treatment. Firstly, its insulinotropic effect is strictly glucose-dependent, which implies that it is unlikely to cause lasting and profound hypoglycaemia [17]. Secondly, it stimulates all steps of insulin biosynthesis as well as insulin gene transcription [18], thereby providing continued supplies of insulin for secretion. In addition, it upregulates the genes for the cellular machinery involved in insulin secretion [19]. And finally and most importantly, GLP-1 has been shown to have trophic effects on b-cells. Not only does it stimulate b-cell proliferation [20, 21], it also enhances the differentiation of new b-cells from progenitor cells in the pancreatic duct epithelium [22]. This indicates that GLP-1 may be capable of providing new b-cells in individuals with an insufficient number of functioning cells [23] such as in type 2 diabetic patients (although it has not yet been established to what extent this process occurs in humans).

In addition to its effects on b-cells, GLP-1 also strongly inhibits glucagon secretion. The importance of this with respect to diabetes treatment is perhaps best illustrated in studies of GLP-1 infusion in patients with insulin-requiring diabetes and no residual b-cell secretory capacity [24]. In these patients, GLP-1 retains substantial glucose-lowering activity, in spite of undetectable C-peptide responses, while strongly inhibiting glucagon secretion. We assume that the glucose-lowering effect is a consequence of the inhibition of glucagon secretion, since we have shown that this effect of GLP-1 is probably mainly due to the inhibition of hepatic glucose production as a result of its combined action on the secretion of the two pancreatic glucoregulatory hormones [25].

Further important effects of GLP-1 include inhibition of gastrointestinal secretion and motility, notably gastric emptying [26]. This effect is also desirable in patients with diabetes because the slower gastric emptying rate reduces postprandial glucose excursions as is evident from the use of another potent gastric inhibitor, amylin, for diabetes treatment [27]. And, finally, GLP-1 inhibits appetite and food intake. This has been demonstrated in normal subjects, obese subjects and subjects with type 2 diabetes [28-30]. The latter effect would support attempts at weight reduction in type 2 diabetic patients and, if effective, would be considered most desirable. It should be noted though that GLP-1 receptor knockout mice do not become obese [8].

Taken together all these effects render GLP-1 unusually attractive as a

therapeutic agent. Indeed, Rachman et al. [31] were able to demonstrate near-normalization of diurnal plasma glucose concentrations during continuous intravenous infusion of GLP-1 in a group of type 2 diabetic patients (Fig. 1).

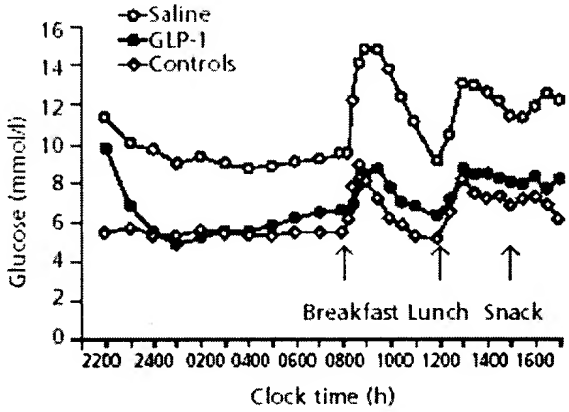

Fig. 1: Mean diurnal plasma glucose concentrations in eight subjects with type 2 diabetes in response to an infusion of saline or a continuous intravenous infusion of GLP-1 (1.2 pmol/kg per min) compared with results obtained in a matched control group. The GLP-1 infusion virtually normalized both fasting glucose concentrations and meal-induced glucose excursions. Reproduced from [31].

However, it appears that simple subcutaneous injections of GLP-1 are ineffective [32]. The reason is that GLP-1 is degraded extremely rapidly after subcutaneous injection. In fact, less than 10% of the peptide survives in an intact bioactive form [33]. The rapid initial degradation is due to the ubiquitously expressed enzyme, dipeptidyl peptidase IV (DPP-IV) [34], which cleaves off the two N-terminal amino acid residues from GLP-1 whereby the molecule is not only inactivated, but may actually be turned into an antagonist at the GLP-1 receptor [35]. Thus it is clear that GLP-1 cannot be immediately employed for clinical treatment of type 2 diabetes. A number of strategies have therefore been explored including: (1) development of small molecule agonists for the GLP-1 receptor; (2) development of DPP-IV-resistant analogues; (3) inhibition of DPP-IV; and (4) continuous subcutaneous infusion of GLP-1.

Small molecules

Such analogues have proven exceedingly difficult to produce. Several pharmaceutical companies have undertaken large screening programmes utilizing the cloned GLP-1 receptor [36], but so far with little success.

DPP-IV-resistant analogues

DPP-IV-resistant analogues of GLP-1 are produced with far greater ease. Consistent with the specificity of DPP-IV for proline or alanine in the penultimate N-terminal position of the substrate peptides [37], analogues of GLP-1 substituted at this position (where GLP-1 has alanine) are generally resistant to DPP-IV [38] and retain a prolonged insulinotropic activity compared with native GLP-1.

A particularly interesting analogue is exendin-4, a peptide of 39 amino acids isolated from the venom of the Gila monster lizard, which has 53% sequence homology to GLP-1. Exendin-4 is a full agonist at the GLP-1 receptor [36] and is resistant to DPP-IV because of the presence of Gly at position 2. Exendin-4 has been demonstrated to potently improve metabolism during prolonged administration in animals with experimental diabetes. Thus, once-daily intraperitoneal administration for 13 weeks in diabetic mice almost normalized blood glucose and reduced HbA1c to near-

normal values [39].
In another study, both blood glucose and HbA1c levels were improved and body weight lowered in up to 42 days of exendin treatment in several experimental animal models of type 2 diabetes [40]. Subsequently, it was demonstrated that 8 weeks' exendin treatment of Zucker fatty rats substantially improved glycaemia, hyperinsulinaemia and body weight [41].
Most recently, Amylin Pharmaceuticals
presented data obtained in patients with type 2 diabetes treated with exendin-4 injections subcutaneously two or three times daily for 28 days in addition to their conventional treatment. A reduction of approximately 1% in HbA1c was obtained, the main side effect being nausea [42].
Another analogue (NN2211) has been developed by Novo Nordisk. Here an acyl chain has been linked to the native GLP peptide, whereby the peptide binds to albumin. Not only does this limit its sensitivity to DPP-IV and delay its absorption from the injection site, the analogue also escapes renal elimination and therefore shows a highly prolonged pharmacokinetic profile in humans with a half-life of about 12 h. Full 24-h coverage with adequate GLP-1 concentrations may therefore be obtained by a single daily injection. Highly encouraging results of the first clinical study with this analogue were recently presented [43].
Thus, the use of DPP-IV-resistant analogues of GLP-1 shows considerable promise.

Inhibitors of DPP-IV

The use of inhibitors of DPP-IV was suggested as a result of the extreme DPP-IV-mediated degradation of GLP-1 in patients with type 2 diabetes [44]. This degradation occurs in the circulation at such high rates that a steady state cannot be established. In this situation the usual parameters of elimination half-life and metabolic clearance rate have no meaning, but calculated as apparent values the half-life is around
1-112 min and the clearance rate exceeds cardiac output by a factor of two [45]. It was demonstrated that with available inhibitors it was
possible to completely protect exogenous and endogenous GLP-1 from DPP-IV-mediated degradation and thereby greatly enhance its insulinotropic activity [46]. Numerous subsequent studies have indicated that administration of orally active DPP-IV inhibitors markedly improved metabolism and glucose regulation in animal models of glucose intolerance. For example, in mice rendered glucose-intolerant by a high-fat diet, the inhibitor valine pyrrolidide almost doubled the plasma levels of undegraded bioactive GLP-1, augmented insulin secretion and virtually normalized the otherwise considerably impaired glucose tolerance (Fig. 2) [47].

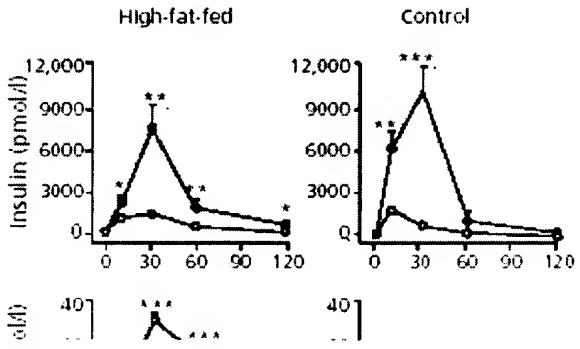

Fig. 2: Plasma concentrations of insulin, glucose and intact, biologically active GLP-1 before and after intragastric administration of glucose with and without the DPP-IV inhibitor valine pyrrolidide in anaesthetized, glucose-intolerant (high-fat-fed) and control mice. Administration of the DPP-IV inhibitor
nearly doubled the concentrations of intact GLP-1, greatly

augmented insulin levels and improved glucose tolerance.
Asterisks indicate the probability level of random difference between the groups, *p < 0.05, **p < 0.01, ***p < 0.001. Reproduced from [46].

Using another inhibitor, Pospisilik et al. [48] reported sustained improvements in glucose tolerance, insulin sensitivity and b-cell mass in Zucker fatty rats (a specific Vancouver strain). Finally, in a human study over 4 weeks, significant improvements in metabolic control resulted from two or three times daily administration of an inhibitor (NVP-DPP728) developed by Novartis [49].
There are many other substrates for DPP-IV than GLP-1, but it seems that the extreme degradation of GLP-1 makes it a preferential target, so that significant protection of GLP-1 can be accomplished without significantly compromising the other functions of DPP-IV. It should be noted that Fischer rats with mutations in the catalytic part of DPP-IV have no malfunctions [50]. The true potential of the treatment with DPP-IV inhibitors is likely to be found in their oral availability and lack of side effects. This means that they can be offered to subjects at risk of developing diabetes, e.g. those with impaired glucose tolerance, a genetic disposition, obesity or mild diabetes. In these subjects it can be expected that because of the amplification of the GLP-1 effects, glucose tolerance will be improved, body weight may be reduced and the b-cells may be protected; the long-term benefits may turn out to be prevention of progression of disease and prevention of complications.

Continuous subcutaneous infusion
One way of providing a constant supply of DPP-IV in a clinically relevant manner is by continuous subcutaneous delivery via a pump. After having demonstrated that such infusions for 48 h have beneficial effects on blood glucose and pancreatic hormone levels as well as appetite [51], we recently embarked on a 6-week study [52]. After a 3-week washout period, 20 patients were allocated to a continuous infusion of either saline or GLP-1 at a rate of 4.8 pmol/kg per min using a Minimed® pump. The patients were evaluated before and after 1 and 6 weeks of treatment. No changes were observed in the saline-treated group, whereas in the GLP-1 group fasting and average plasma glucose concentrations were lowered by approximately 5 mmol/l, HbA1c decreased by 1.2%, free fatty acids were significantly lowered, and the patients had a significant weight loss of approximately 2 kg. In addition, insulin sensitivity as determined by a hyperinsulinaemic-euglycaemic clamp almost doubled, and insulin secretion capacity (measured using a 30 mmol/l glucose clamp + arginine) greatly improved. There was no significant difference between the results obtained after 1 and 6 weeks' treatment, but there was a tendency towards further improvement in plasma glucose as well as in insulin secretion. There were very few side effects and no differences between saline- and GLP-1-treated patients in this respect.
These results not only demonstrate that insulin pumps can be used to provide clinically relevant GLP-1 treatment, they also demonstrate the power of GLP-1 treatment, resulting in marked metabolic improvements, improvements in pancreatic endocrine function and reduction in protein

glycation. Thus, we may conclude that diabetes treatment based on GLP-1 is an unusually interesting alternative to the existing treatment modalities. In addition, a combination of GLP-1 with other treatments may be beneficial: a combination of metformin and GLP-1 therapy has been shown to have additive effects [53]. However, the most exciting aspect is the possibility that GLP-1 treatment – perhaps because of its unique trophic effects on the pancreas – may halt the progression of disease that inevitably seems to accompany conventional treatment [54].

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